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(54) Title: NOVEL COMPONENT OF VON HIPPEL-LINDAU TUMOR SUPPRESSOR COMPLEX AND SCF UBIQUITIN LIGASE			
(57) Abstract			
<p>Rbx1, an evolutionarily conserved Cullin-interacting RING-H2 finger protein, has been discovered. Mammalian Rbx1 has been identified as a component of the CUL2-containing VHL complex. An Rbx1 homolog from <i>S. cerevisiae</i> has also been identified as a subunit and activator of the Cdc53-containing SCF^{Cdc4} ubiquitin ligase required for ubiquitination of the cdk inhibitor Sic1 and for the G1/S cell cycle transition in yeast, providing a link between the multiprotein VHL tumor suppressor complex and cellular ubiquitination. The Rbx1 protein acts as a cellular marker useful (1) in detecting a possible predisposition to certain carcinomas, (2) as a molecular target for treating those carcinomas therapeutically, (3) as a target for inhibition by drugs that manipulate the growth of cells, and (4) as a research tool to better understand the various complex mechanisms of cell ubiquitination, binding of certain activator proteins, fibronectin deposition and other aspects of the cellular division process.</p>			

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NOVEL COMPONENT OF VON HIPPEL-LINDAU
TUMOR SUPPRESSOR COMPLEX AND SCF UBIQUITIN LIGASE

TECHNICAL FIELD OF THE INVENTION

The invention relates to a bio-affecting and body treating composition and to a biological
5 diagnostic agent.

BACKGROUND OF THE INVENTION

The von Hippel-Lindau (VHL) tumor suppressor gene on chromosome 3p25.5 is mutated
in the majority of sporadic clear cell renal carcinomas and in VHL disease, an autosomal
dominant familial cancer syndrome that predisposes affected individuals to a variety of tumors
10 including clear cell renal carcinomas, cerebellar hemangioblastomas and hemangiomas, retinal
angiomas, and pheochromocytomas. (Linehan, *et al.* 1995. "Identification of the von Hippel-
Lindau (VHL) gene. Its role in renal cancer," *JAMA* 273:564-570; Gnarr, *et al.* 1996.
"Molecular cloning of the von Hippel-Lindau tumor suppressor gene and its role in renal
carcinoma," *Biochim Biophys Acta* 1242:201-210; and Kaelin, W.G. and E.R. Maher. 1998.
15 "The VHL tumor-suppressor gene paradigm," *Trends Genet* 14:423-426). The VHL protein is
expressed in most tissues and cell types and appears to perform multiple functions, including
general repression of hypoxia-inducible genes (Iliopoulos, *et al.* 1996. "Negative regulation of
hypoxia-inducible genes by the von Hippel-Lindau protein," *Proc Natl Acad Sci USA*
93:10595-10599; Gnarr, *et al.* 1996. Post-transcriptional regulation of vascular endothelial
20 growth factor mRNA by the product of the VHL tumor suppressor gene," *Proc Natl Acad Sci*
USA 93:10589-10594; and Siemeister, *et al.* 1996. "Reversion of deregulated expression of
vascular endothelial growth factor in human renal carcinoma cells by von Hippel-Lindau tumor
suppressor protein," *Cancer Res* 56:2299-2301); regulation of p27 protein stability (Pause, *et al.*
1998. "The von Hippel-Lindau tumor suppressor gene is required for cell cycle exit upon serum
25 withdrawal," *Proc Natl Acad Sci USA* 95:993-998; and Kim, *et al.* 1999. "Recombinant
adenovirus expressing Von Hippel-Lindau-mediated cell cycle arrest is associated with the
induction of cyclin-dependent kinase inhibitor p27Kipl," *BBRC* 253:672-677); and regulation of
the assembly of extracellular fibronectin matrix (Ohh, *et al.* 1998. "The von Hippel-Lindau
tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix,"
30 *Mol Cell* 1:959-968).

In all cell types examined, the VHL protein is found in a multiprotein complex that
includes the ubiquitin-like Elongin B protein, and Elongin C and the cullin CUL2, which share
sequence similarity with the Skp1 and Cdc53 components of the Skp1-Cdc53p-F-box protein
(SCF) ubiquitin ligase complex, respectively. (Kibel, *et al.* 1995. "Binding of the von Hippel-
35 Lindau tumor suppressor protein to Elongin B and C," *Science* 269:1444-1446; Duan, *et al.*

1995. "Inhibition of transcription elongation by the VHL tumor suppressor protein," *Science* 269:1402-1406; Pause, *et al.* 1997. "The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins," *Proc Natl Acad Sci USA* 94:2156-2161; and Lonergan, *et al.* 1998. "Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing Elongins B/C and Cul2," *Mol Cell Biol* 18:732-741). Elongins B and C form a stable subcomplex that interacts with a short BC-box motif in the VHL protein and bridges its interaction with CUL2. (Pause, *et al.* 1997. *Proc Natl Acad Sci USA* 94:2156-2161; Lonergan, *et al.* 1998. *Mol Cell Biol* 18:732-741). A large fraction of VHL mutations found in sporadic clear cell renal carcinomas and in VHL kindreds result in mutation or deletion of the BC-box, disruption of the VHL complex, and deregulation of hypoxia-inducible gene expression, p27 protein stability, and bironectin matrix assembly. (Gnarra, *et al.* 1996. *Biochim Biophys Acta* 1242:201-210; Pause, *et al.* 1997. *Proc Natl Acad Sci USA* 94:2156-2161; Lonergan, *et al.* 1998. *Mol Cell Biol* 18:732-741; Ohh, *et al.* 1998. *Mol Cell* 1:959-968; and Kaelin, W.G. and E.R. Maher. 1998. *Trends Genet* 14:423-426).

Despite information currently available regarding the multiprotein complex that includes the VHL protein, Elongin B, Elongin C, and CUL2, not all components of this complex and the binding relationship of these components to one another have been elucidated. There is a continuing need to characterize yet unknown components of the complex and their interaction with other components to further the understanding of the complex and its relationship to disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A - 1C describe the co-purification of the VHL complex with Rbx1 from rat liver cytosol.

Fig. 1A is a schematic showing the various steps used in the purification of the VHL complex, wherein P-cell = phosphocellulose P11.

Fig. 1B is a chromatography of Rbx1 with the VHL complex, wherein VHL = von Hippel-Lindau protein; CUL2 = CUL2 protein or cullin; EloB = Elongin B; EloC = Elongin C. Aliquots of column fractions from the MonoQ column were subjected to 12% SDS-polyacrylamide gel electrophoresis, and proteins were detected by silver staining.

Fig. 1C depicts a 5-20% SDS-polyacrylamide gel of sample used for peptide sequencing.

Fig. 2 is a chart showing the alignment of predicted Rbx1 protein sequences from human, mouse, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* with APC11 from *S. cerevisiae*, wherein DROS = *Drosophila melanogaster*; ELEGANS =

Caenorhabditis elegans; and YEAST = *Saccharomyces cerevisiae*. The alignment was generated using the MACAW program. (Schuler, *et al.* 1991. "A workbench for multiple alignment construction and analysis," *Proteins; Struct Funct Genet* 9:180-190). Dark shading indicates positions of identity between Rbx1 proteins from different species and positions of identity between the Rbx1 and APC11 proteins. Grey shading indicates position of similarity.

Fig. 3A - 3D depict the reconstitution of Rbx1- containing complexes.

Fig. 3a demonstrates Rbx1 forming complexes with VHL and CUL2 in the presence of Elongin BC. Lysates from Sf21 cells expressing the indicated viruses were immunoprecipitated with anti-FLAG or anti-MYC antibodies, and immunoprecipitated proteins were detected by immunoblotting.

Fig. 3B demonstrates independent association of Rbx1 with Elongin BC, and VHL. Lysates from Sf21 cells expressing the indicated viruses were immunoprecipitated with anti-HPC4, anti-FLAG, or anti-MYC antibodies, and immunoprecipitated proteins were detected by immunoblotting.

Fig. 3C demonstrates *in vitro* binding of recombinant Rbx1, VHL, and Elongin BC. Proteins expressed in and purified from *E. coli* were mixed together in the combinations indicated, renatured by dilution and dialysis, and immunoprecipitated with anti-HPC4. Immunoprecipitated proteins were detected by immunoblotting with the indicated antibodies.

Fig. 3D demonstrates independent association of Rbx1 with Cul1, Cul2, and Cdc53. Lysates from Sf21 cells expressing the indicated viruses were immunoprecipitated with anti-MYC antibodies, and immunoprecipitated proteins were detected by immunoblotting.

Fig. 4A - 4C depict various activities associated with the presence or absence of Rbx1.

Fig. 4A demonstrates that binding of Rbx1 protein to endogenous yeast Cdc53 correlates with function. The upper panel shows phenotypes of *rbx1*Δ cells expressing wild type or mutant mammalian Rbx1 (mRbx1) protein. As shown in the lower panel, lysates from cells expressing wild type and mutant mammalian MYC-Rbx1 proteins in either the *rbx1* deletion strain (deleted) or in the parental strain MCY453 (wild type) were subjected to immunoprecipitation with anti-MYC antibodies. Immunoprecipitated proteins were detected by immunoblotting with anti-MYC or anti-Cdc53 antibodies.

Fig. 4B demonstrates that Sic1 protein accumulates in Rbx1-depleted cells. *Rbx1*ΔpGAL-mrbx1 (M4) cells were grown to an OD600 of 1 in galactose-containing medium and then shifted into glucose medium. Cells were harvested after 8 hours of growth in glucose, and cell lysates were analyzed by immunoblotting with anti-MYC and two different anti-Sic1 antibodies.

Fig. 4C demonstrates morphological changes associated with Rbx1-depletion. *Rbx1* Δ pGAL-mrbx1 (M4) cells were grown in galactose (gal) or for 8 hours after glucose shift (glu) prior to fixation and staining. Nuclear morphology was visualized by DAPI (4,6-diamidino-2-phenylindole) staining. (Panels A and C, DIC; panels B and D, DAPI).

Fig. 5 shows a diagram of a SCF ubiquitin ligase complex and a VHL ubiquitin ligase complex and illustrates the ubiquitin ligases of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The terms "Ring box protein" and "Rbx1" as used herein refers to proteins that are components of the von Hippel-Lindau tumor suppressor complex. The terms refer to proteins obtained from any eukaryotic species, particularly mammalian species such as bovine, porcine, murine, equine, and human, and from any source whether natural, synthetic, semi-synthetic, or recombinant. The terms encompass polypeptides or proteins having less than the complete amino acid sequence and biologically active variants and gene products.

The term "naturally occurring" as used herein means an endogenous or exogenous protein isolated and purified from animal tissue or cells.

The term "isolated and purified" as used herein means a protein that is essentially free of association with other proteins or polypeptides, e.g., as a naturally occurring protein that has been separated from cellular and other contaminants by the use of antibodies or other methods or as a purification product of a recombinant host cell culture.

The term "biologically active" as used herein means a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule.

The term "nucleotide sequence" as used herein means a polynucleotide molecule in the form of a separate fragment or as a component of a larger nucleic acid construct that has been derived from DNA or RNA isolated at least once in substantially pure form (i.e., free of contaminating endogenous materials) and in a quantity or concentration enabling identification, manipulation, and recovery of its component nucleotide sequences by standard biochemical methods. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal non-translated sequences, or introns that are typically present in eukaryotic genes. Sequences of non-translated DNA may be present 5' or 3' from an open reading frame where the same do not interfere with manipulation or expression of the coding region.

The term "nucleic acid molecule" as used herein means RNA or DNA, including cDNA, single or double stranded, and linear or covalently closed molecules. A nucleic acid molecule

may also be genomic DNA corresponding to the entire gene or a substantial portion thereof or to fragments and derivatives thereof. The nucleotide sequence may correspond to the naturally occurring nucleotide sequence or may contain single or multiple nucleotide substitutions, deletions and/or additions including fragments thereof. All such variations in the nucleic acid molecule retain the ability to encode a biologically active protein when expressed in the appropriate host or a biologically active fragment thereof. The nucleic acid molecule of the present invention may comprise solely the nucleotide sequence encoding a protein or may be part of a larger nucleic acid molecule that comprises the gene for [extends to the gene for the protein. The non-protein encoding sequences in a larger nucleic acid molecule may include vector, promoter, terminator, enhancer, replication, signal sequences, or non-coding regions of the gene.

The term "variant" as used herein means a polypeptide substantially homologous to a naturally occurring protein but which has an amino acid sequence different from the naturally occurring protein (human, bovine, ovine, porcine, murine, equine, or other eukaryotic species) because of one or more deletions, insertions, derivations, or substitutions. The variant amino acid sequence preferably is at least 40% identical to a naturally occurring amino acid sequence but is most preferably at least 70% identical. Variants may comprise conservatively substituted sequences wherein a given amino acid residue is replaced by a residue having similar physiochemical characteristics. Conservative substitutions are well known in the art and include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Conventional procedures and methods can be used for making and using such variants. Other such conservative substitutions such as substitutions of entire regions having similar hydrophobicity characteristics are well known. Naturally occurring variants are also encompassed by the present invention. Examples of such variants are proteins that result from alternate mRNA splicing events or from proteolytic cleavage of the protein that leave the protein biologically active and capable of performing its biological function. Alternate splicing of mRNA may yield a truncated but biologically active protein. Variations attributable to proteolysis include differences in the N- or C-termini upon expression in different types of host cells due to proteolytic removal of one or more terminal amino acids from the protein.

The term "substantially the same" as used herein means nucleic acid or amino acid sequences having sequence variations that do not materially affect the nature of the protein, *i.e.*, the structure and/or biological activity of the protein. With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to

conserved sequences governing expression and refers primarily to degenerate codons encoding the same amino acid or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The term "percent identity" as used herein means comparisons among amino acid sequences as defined in the UWGCG sequence analysis program available from the University of Wisconsin. (Devereaux *et al.*, *Nucl. Acids Res.* 12: 387-397 (1984)).

This invention is not limited to the particular methodology, protocols, cell lines, vectors, and reagents described because these may vary. Further, the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention. As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise, *e.g.*, reference to "a host cell" includes a plurality of such host cells.

Because of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding Rbx1 or other Ring box proteins referred to herein may be produced. Some of these sequences will be highly homologous and some will be minimally homologous to the nucleotide sequences of any known and naturally occurring gene. The present invention contemplates each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring Rbx1 and all such variations are to be considered as being specifically disclosed.

Unless defined otherwise, all technical and scientific terms and any acronyms used herein have the same meanings as commonly understood by one of ordinary skill in the art in the field of the invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred methods, devices, and materials are described herein.

All patents and publications mentioned herein are incorporated herein by reference to the extent allowed by law for the purpose of describing and disclosing the proteins, enzymes, vectors, host cells, and methodologies reported therein that might be used with the present invention. However, nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

The Invention

The present invention provides isolated and purified biologically active cullin-interacting RING-H2 finger proteins ("Ring box proteins") that are a component of the von Hippel-Lindau tumor suppressor complex and of SCF ubiquitin ligase complexes, nucleic acid molecules that
5 encode Ring box proteins, expression vectors having DNA that encode Ring box proteins, host cells that have been transfected or transformed with expression vectors having DNA that encode Ring box proteins, methods for producing recombinant Ring box proteins by culturing host cells that have been transfected or transformed with expression vectors having DNA that encode Ring box proteins, isolated and purified recombinant Ring box proteins, complexes and methods that
10 use Ring box proteins to screen for therapeutic agents, methods for diagnosing the predisposition of a patient to certain carcinomas, methods for treating any of several enumerated carcinomas or augmenting metabolically deficient systems in humans and other animals, and methods for evaluating the effectiveness of a therapeutic treatment for Ring box associated carcinomas. In particular, the present invention provides a new Ring box protein designated
15 Rbx1.

Isolation and Purification of Rbx1

The endogenous VHL complex from rat liver was first purified by the procedures given below and outlined in Fig. 1A. The VHL complex was purified from a post-nuclear supernatant prepared from the livers of 360 male Sprague-Dawley rats and fractionated by $(\text{NH}_4)_2\text{SO}_4$
20 precipitation as described previously. (Conaway, *et al.* 1996. "Purification of RNA polymerase II general transcription factors from rat liver," *Meth Enzymol* 273:194-207). Fractions containing the VHL complex were identified by Western blotting using IG32, a monoclonal against the VHL protein. (Kibel, *et al.* 1995. "Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C," *Science* 269:1444-1446). The 0 to 38% $(\text{NH}_4)_2\text{SO}_4$
25 fraction was resuspended in Buffer A [20 mM Hepes-NaOH (pH 7.9), 0.5 mM EDTA, 1 mM DTT, 10% (v/v) glycerol] containing 0.5 mM PMSF, 10 $\mu\text{g/ml}$ antipain, and 10 $\mu\text{g/ml}$ leupeptin and was brought to a conductivity equivalent to that of Buffer A containing 100 mM KCl by a 3 hour dialysis against Buffer A containing 0.5 mM PMSF and dilution with the same buffer. The dialysate was centrifuged for 30 min at 28,000 x g, and the supernatant was gently mixed for
30 ~45 minutes with 800 ml of phosphocellulose (P11, Whatman) pre-equilibrated in Buffer A containing 100 mM M KCl and 0.5 mM PMSF. The slurry was then filtered at 500 ml/hr in a 10.5-cm diameter column. The phosphocellulose flow through fraction was gently mixed for ~45 minutes with 250 ml Toyopearl DEAE-650M (TosoHaas) pre-equilibrated in Buffer C [40 mM Tris-HCl (pH 7.9), 0.5 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol] containing 80 mM

KCl. The slurry was filtered at 150 ml/hr in a 5.0-cm diameter column and then washed at the same flow rate with Buffer C containing 80 mM KCl. The column was eluted stepwise at 250 ml/hr with Buffer A containing 220 mM KCl, and 50 ml fractions were collected. Fractions containing the VHL protein were concentrated by 0.3 mg/ml $(\text{NH}_4)_2\text{SO}_4$ precipitation, resuspended in 10 ml of Buffer A containing 10 $\mu\text{g/ml}$ antipain and 10 $\mu\text{g/ml}$ leupeptin, and dialyzed against Buffer A containing 300 mM KCl to a conductivity equivalent to that of Buffer A containing 500 mM $(\text{NH}_4)_2\text{SO}_4$. Following centrifugation for 15 min at 12,000 x g, the resulting supernatant was applied at 20 ml/hr to a 500 ml, 2.6-cm diameter Ultrogel AcA 34 gel filtration column (IBF Biotechnics) pre-equilibrated in Buffer A containing 400 mM KCl. The column was eluted at 20 ml/hr, and 10 ml fractions were collected. Fractions containing the VHL protein, which eluted with an apparent native molecular mass between 330 kDa and 200 kDa, were diluted with an equal volume of Buffer E [40 mM Hepes-NaOH (pH 7.9), 0.1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol] containing 2.0 M $(\text{NH}_4)_2\text{SO}_4$. Following centrifugation for 20 min at 60,000 x g, the resulting supernatant was applied to a Spherogel TSK phenyl 5-PW column (21.5- x 150-mm, Beckman Instruments) pre-equilibrated in Buffer E containing 1.0 M $(\text{NH}_4)_2\text{SO}_4$. The column was eluted at 5 ml/min with a 250 ml linear gradient from 1.0 M $(\text{NH}_4)_2\text{SO}_4$ in Buffer E to Buffer E, and 5 ml fractions were collected. Fractions containing the VHL protein, which eluted between 170 mM and 80 mM $(\text{NH}_4)_2\text{SO}_4$, were pooled and dialyzed against Buffer C to a conductivity equivalent to that of Buffer C containing 60 mM KCl. Following centrifugation for 20 min at 60,000 x g, the resulting supernatant was applied to a Bio-Gel SEC DEAE 5-PW column (7.5- x 75-mm, Bio-Rad) pre-equilibrated in Buffer C containing 60 mM KCl. The column was eluted at 0.8 ml/min with a 40 ml linear gradient from 60 mM to 250 mM KCl in Buffer C, and 0.7 ml fractions were collected. Fractions containing the VHL protein, which eluted between 120 mM and 140 mM KCl, were pooled and brought to 5 mM potassium phosphate. Following centrifugation for 20 min at 60,000 x g, the resulting supernatant was applied to a crystalline hydroxylapatite Bio-Scale CHT-1 column (7- x 52-mm, Bio-Rad) pre-equilibrated in Buffer P [5 mM potassium phosphate (pH 7.6), 0.1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol]. The column was eluted at 0.6 ml/min with a 24 ml linear gradient from 50 mM to 400 mM potassium phosphate (pH 7.6) in Buffer P, and 0.3 ml fractions were collected. Fractions containing the VHL protein, which eluted between 50 mM and 80 mM potassium phosphate, were pooled and diluted with Buffer C containing 50 mM KCl to a conductivity equivalent to that of Buffer C in 80 mM KCl. Following centrifugation for 20 min at 60,000 x g, the resulting supernatant was applied to a MonoQ column (5- x 50-mm, Pharmacia) pre-equilibrated in Buffer C containing 80 mM KCl.

The column was eluted at 0.4 ml/min with a 12 ml linear gradient from 80 mM to 300 mM KCl in Buffer C, and 0.2 ml fractions were collected. Fractions containing the VHL protein eluted between 180 mM and 200 mM KCl.

As shown in Fig. 1B and 1C, greater than 90% of the detectable VHL protein in liver homogenates copurified with CUL2, Elongin B, Elongin C, and a small polypeptide with an apparent molecular mass of ~16 kDa. The identities of the VHL, CUL2, Elongin B, and Elongin C polypeptides were confirmed by Western blotting and/or peptide sequencing. On-line ion trap HPLC/MS/MS peptide sequencing using the method described below (Eng, *et al.* 1994. *J Am Soc Mass Spectrom* 5:976 and Chittum *et al.* 1998. *Biochemistry* 37:10866) of the ~16 kDa protein excised from the SDS polyacrylamide gel shown in Fig. 1C revealed that it was a novel RING-H2 finger protein, designated "Rbx1" or "Ring-box protein."

Sequencing of Rbx1

The VHL tumor suppressor complex was fractionated by 13% SDS-polyacrylamide gel electrophoresis. Proteins were visualized by staining the gel with Coomassie blue, excised, and subjected to in-gel reduction, S-carboxyamidomethylation, and tryptic digestion. Using 10% of the digestion mixture, peptide sequences were determined in a single run by microcapillary reversed-phase chromatography coupled to the electrospray ionization source of a quadrupole ion trap mass spectrometer (Finnigan LCQ). The ion trap's online data-dependent scans allowed the automatic acquisition of high resolution spectra to determine charge state and exact mass, and tandem mass spectrometry spectra for sequence information. The relative collision energy was 35%, and isolation width was 2.5 Dalton. Searches of the EST database performed using TLASTIN algorithm identified human and mouse ESTs that encoded the peptide sequences NHMDLCIECQAN, QVCPDNREWEFQK, WNAVAL and WLK which were determined by ion trap mass spectrometry of the 16 kDa polypeptide that copurified with the VHL complex.

The identification was facilitated with the algorithm SEQUEST (Eng, *et al.* 1994. *J Am Soc Mass Spectrom* 5:976) and by programs developed in the Harvard Microchemistry Facility (Chittum *et al.* 1998. *Biochemistry* 37:10866). I.M.A.G.E. Consortium cDNA clones (I.M.A.G.E. Consortium: an integrated molecular analysis of genomes and their expression," *Genomics* 33:151-152) encoding the complete 108 amino acid long ORFs of human (H71993) and mouse (W66989 and AA260889) Rbx1 were obtained from Research Genetics, Huntsville, Alabama, and the nucleotide sequences of both strands were determined. Human and mouse cDNAs encoded identical polypeptides of 108 amino acids. The amino acid sequence for human and mouse Rbx1 is shown in Fig. 2 and in SEQ ID NO:1. The nucleotide sequence for the human Rbx1 DNA is shown in nucleotides 7-333 of SEQ ID NO:3 and the nucleotide sequence

for the murine Rbx1 DNA is shown in nucleotides 18-344 of SEQ ID NO:5, inclusive of the stop codon. Nucleotides 1-6 and 1-17 are 5' untranslated regions respectively and 334-508 and 345-504 are 3' untranslated regions respectively.

As shown in Fig. 2, Rbx1 is highly homologous to *D. melanogaster* ORF 115C2.11, *C. elegans* ORF ZK287.5, and *S. cerevisiae* ORF YOL133w. The amino acid sequence for *Saccharomyces cerevisiae* Rbx1 is shown in SEQ ID NO:2 and the nucleotide sequence for the *Saccharomyces cerevisiae* Rbx1 DNA is shown in nucleotides 4-369 of SEQ ID NO:4, inclusive of the stop codon. Comparison of the deduced amino acid sequences demonstrates that the proteins are highly homologous with about an 80 percent identity and that the proteins are substantially the same. In addition, Rbx1 exhibits significant sequence similarity with *Saccharomyces cerevisiae* Anaphase-Promoting Complex subunit APC11 (Zachariae, *et al.* 1998. "Mass spectrometric analysis of the anaphase-promoting complex from yeast: Identification of a subunit related to cullins," *Science* 279:1216-1219).

Because of the degeneracy of the genetic code, a DNA sequence may vary from that shown in SEQ ID NO:3 and still encode a Rbx1 protein having the amino acid sequence shown in SEQ ID NO:1. Such variant DNA sequences may result from silent mutations, *e.g.*, occurring during PCR amplification, or may be the product of deliberate mutagenesis of a native sequence. The invention, therefore, provides equivalent isolated DNA sequences encoding biologically active Rbx1 selected from: (a) the coding region of a native Rbx1 gene; (b) cDNA comprising the nucleotide sequence presented in SEQ ID NO:3; (c) DNA capable of hybridization to the native Rbx1 gene under moderately stringent conditions and which encodes biologically active Rbx1; and (d) DNA which is degenerate as a result of the genetic code to a DNA defined in (a), (b), or (c) and which encodes biologically active Rbx1. Rbx1 encoded by such DNA equivalent sequences are encompassed by the invention.

Recombinant Expression for Rbx1

Isolated and purified recombinant Rbx1 is provided according to the present invention by incorporating the corresponding DNA into expression vectors and expressing the DNA in a suitable host cell to produce the protein.

Expression Vectors

Recombinant expression vectors containing a nucleic acid sequence encoding the protein can be prepared using well known techniques. The expression vectors include a DNA sequence operably linked to suitable transcriptional or translational regulatory nucleotide sequences such as those derived from mammalian, microbial, viral, or insect genes. Examples of regulatory sequences include transcriptional promoters, operators, enhancers, mRNA ribosomal binding

sites, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are "operably linked" when the regulatory sequence functionally relates to the DNA sequence for the appropriate protein. Thus, a promoter nucleotide sequence is operably linked to a Rbx1 DNA sequence if the promoter nucleotide sequence controls the transcription of the appropriate DNA sequence.

The ability to replicate in the desired host cells, usually conferred by an origin of replication and a selection gene by which transformants are identified, may additionally be incorporated into the expression vector.

In addition, sequences encoding appropriate signal peptides that are not naturally associated with Rbx1 can be incorporated into expression vectors. For example, a DNA sequence for a signal peptide (secretory leader) may be fused in-frame to the protein sequence so that the protein is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells enhances extracellular secretion of the appropriate polypeptide. The signal peptide may be cleaved from the polypeptide upon secretion of protein from the cell.

Host Cells

Suitable host cells for expression of Rbx1 include prokaryotes, yeast, archae, and other eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are well known in the art, *e.g.*, Pouwels *et al.* Cloning Vectors: A Laboratory Manual, Elsevier, New York (1985). The vector may be a plasmid vector, a single or double-stranded phage vector, or a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. The vectors, in the case of phage and viral vectors also may be and preferably are introduced into cells as packaged or encapsulated virus by well known techniques for infection and transduction. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells. Cell-free translation systems could also be employed to produce the protein using RNAs derived from the present DNA constructs.

Prokaryotes useful as host cells in the present invention include gram negative or gram positive organisms such as *E. coli* or *Bacilli*. In a prokaryotic host cell, a polypeptide may include a N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant Rbx1 polypeptide. Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β -lactamase and the lactose promoter system.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those
5 derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. To construct an expression vector using pBR322, an appropriate promoter and a DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine
10 Chemicals, Uppsala, Sweden), pGEM1 (Promega Biotec, Madison, Wisconsin, USA), and the pET (Novagen, Madison, Wisconsin, USA) and pRSET (Invitrogen Corporation, Carlsbad, California, USA) series of vectors (Studier, F.W., *J. Mol. Biol.* 219: 37 (1991); Schoepfer, R. *Gene* 124: 83 (1993)).

Promoter sequences commonly used for recombinant prokaryotic host cell expression
15 vectors include T7, (Rosenberg, A.H., Lade, B. N., Chui, D-S., Lin, S-W., Dunn, J. J., and Studier, F. W. (1987) *Gene* (Amst.) 56, 125-135), β -lactamase (penicillinase), lactose promoter system (Chang *et al.*, *Nature* 275:615, (1978); and Goeddel *et al.*, *Nature* 281:544, (1979)), tryptophan (trp) promoter system (Goeddel *et al.*, *Nucl. Acids Res.* 8:4057, (1980)), and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory,
20 p. 412 (1982)).

Yeasts useful as host cells in the present invention include those from the genus *Saccharomyces*, *Pichia*, *K.* *Actinomyces* and *Kluyveromyces*. Yeast vectors will often contain an origin of replication sequence from a 2 μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription
25 termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.* 255:2073, (1980)) or other glycolytic enzymes (Holland *et al.*, *Biochem.* 17:4900, (1978)) such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-
30 phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Fleer *et al.*, *Gene*, 107:285-195 (1991). Other suitable promoters and vectors for yeast and yeast transformation protocols are well known in the art.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen *et al.*, *Proc Natl Acad Sci USA*, 75:1929 (1978). The Hinnen protocol selects for Trp^{sup.} transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 µg/ml adenine, and 20 µg/ml uracil.

Mammalian or insect host cell culture systems well known in the art could also be employed to express recombinant Rbx1, *e.g.*, Baculovirus systems for production of heterologous proteins in insect cells (Luckow and Summers, *Bio/Technology* 6:47 (1988)) or Chinese hamster ovary (CHO) cells for mammalian expression may be used. Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell, *e.g.*, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication. Exemplary expression vectors for use in mammalian host cells are well known in the art.

Rbx1 and other Ring box proteins may, when beneficial, be expressed as a fusion protein that has the Ring box protein attached to a fusion segment. The fusion segment often aids in protein purification, *e.g.*, by permitting the fusion protein to be isolated and purified by affinity chromatography. Fusion proteins can be produced by culturing a recombinant cell transformed with a fusion nucleic acid sequence that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of the protein. Preferred fusion segments include, but are not limited to, glutathione-S-transferase, β-galactosidase, a poly-histidine segment capable of binding to a divalent metal ion, and maltose binding protein.

Expression and Recovery

According to the present invention, isolated and purified Rbx1 may be produced by the recombinant expression systems described above. The method comprises culturing a host cell transformed with an expression vector comprising a DNA sequence that encodes the protein under conditions sufficient to promote expression of the protein. The protein is then recovered from culture medium or cell extracts, depending upon the expression system employed. As is known to the skilled artisan, procedures for purifying a recombinant protein will vary according to such factors as the type of host cells employed and whether or not the recombinant protein is

secreted into the culture medium. When expression systems that secrete the recombinant protein are employed, the culture medium first may be concentrated. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, e.g., a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. Also, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Further, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media (e.g., silica gel having pendant methyl or other aliphatic groups), ion exchange-HPLC (e.g., silica gel having pendant DEAE or sulfopropyl (SP) groups), or hydrophobic interaction-HPLC (e.g., silica gel having pendant phenyl, butyl, or other hydrophobic groups) can be employed to further purify the protein. Some or all of the foregoing purification steps, in various combinations, are well known in the art and can be employed to provide an isolated and purified recombinant protein.

Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange, affinity purification, or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

In another aspect, the present invention provides a protein complex useful for screening for potential therapeutic agents that would interfere with or augment Rbx1-dependent stimulation of addition of ubiquitin or a ubiquitin-like protein to any substrate targeted for modification by SCF complexes. The complex contains a cofactor protein and one or more proteins selected from the group consisting of a cullin protein, a substrate recognition protein, and a linker protein. Additional components, e.g., ATP, may be added to the solution or composition containing the complex to facilitate complex formation and utilization.

In a preferred embodiment, the complex is a ubiquitin ligase protein complex that contains cullin proteins; substrate recognition proteins; linker proteins; and cofactor proteins. The complex preferably has one protein from each group but can have more than one protein from each group if required or preferred. Preferably, the complex is formed from the proteins but the solution or compositions containing the complex may contain additional components if needed to facilitate complex formation and utilization. For example, solutions or compositions

containing the complex can comprise various combinations of (1) Skp1, Elongin C, or other linker protein; (2) β -TRCP, Cdc4, Grr1, or other F-box substrate recognition protein or VHL or other Elongin C-Binding substrate recognition protein; (3) CUL1, Cdc53, CUL2, or other cullin protein; (4) Cdc34, UBC5C, or another E2 ubiquitin conjugating enzyme; (5) phosphorylated or
5 other appropriately modified substrate; (6) E1 ubiquitin activating enzyme; (7) ATP; and (8) ubiquitin, GST-ubiquitin, GST-Ubiquitin^{RA}, ubiquitin derivatives, or a ubiquitin-like protein such as SUMO, NEDD8, or Rub 1.

Preferably, the complex is a ubiquitin ligase protein complex that contains a cullin protein, a substrate recognition protein, a linker protein, and a cofactor protein. Most preferably,
10 the complex is an isolated and purified ubiquitin ligase protein complex comprising such proteins.

Generally, the cullin proteins of the present invention are proteins selected from the group consisting of Cdc53, Cullin 1 (CUL1), Cullin 2 (CUL2), Cullin 3 (CUL3), Cullin 4A (CUL4A), Cullin 4B (CUL4B), and Cullin 5 (CUL5), preferably CUL1 or CUL2. The substrate
15 recognition proteins are selected from the group consisting of F-box proteins such as β -TRCP (HOS), Cdc4, Grr1, and other members of this protein family and VHL and other Elongin C binding proteins, preferably F-box proteins or VHL. The linker proteins are selected from the group consisting of Skp1 or Elongin C. Many such cullin, substrate recognition, and linker proteins are known in the art and can be used in the present invention. The cofactor proteins are
20 the Ring box proteins of the present invention, preferably Rbx1.

Figure 5 shows a diagram of SCF and VHL ubiquitin ligase complexes illustrating the ubiquitin ligases of the present invention. The cullin protein is Cdc53/Cul1 in the SCF complex and CUL2 in the VHL complex, the substrate recognition protein is a F-box protein in the SCF complex and the VHL protein in the VHL complex, and the linker protein is Skp1 in the SCF
25 complex and Elongin C in the VHL complex. The VHL complex also contains Elongin B. The cofactor protein Rbx1 binds to the other proteins and activates the ubiquitin ligase. The SCF ubiquitin ligase targets such substrate proteins as Sic1, cyclins, β -catenin, NF- κ B inhibitors such as I κ B α , and the like, and VHL ubiquitin ligase complex targets such substrate proteins as hypoxia-inducible factor 1 (HIF1) and hypoxia-inducible factor 2 (HIF2)

30 The present invention also provides methods for screening for potential therapeutic agents that would interfere with or augment Rbx1-dependent stimulation of addition of ubiquitin or a ubiquitin-like protein to any substrate including those targeted for modification by SCF of VHL-containing complexes. The methods require forming a complex *in vitro* that contains a cofactor protein and one or more proteins selected from the group consisting of a cullin protein,

a substrate recognition protein, and a linker protein; adding a test compound to interact with the complex; and determining if the complex remains intact or is disrupted by the compound. If the complex is disrupted, the compound is likely to be a therapeutic agent useful for the treatment of the corresponding disease, e.g., cancer or inflammatory disease. For example, a complex can be formed *in vitro* between a Cdc53 and Rbx1. A test compound can be added to interact with the complex. An assay, e.g., SDS-PAGE and immunoblotting or other known techniques, can be conducted to determine if the complex remains intact or is disrupted. SCF ubiquitin ligases control the stability of proteins including but not limited to cyclins and cyclin dependent kinase inhibitors, which regulate the cell cycle, and I κ B, which regulates inflammatory processes. If the complex is disrupted, the compound is a likely candidate for an anti-cancer agent or an anti-inflammatory drug.

Preferably, the complex used in the methods is the ubiquitin ligase protein complex described herein, most preferably an isolated and purified ubiquitin ligase protein complex.

In similar assays, Rbx1 can be used to screen for agents which augment or inhibit activity of other Cullin-containing ubiquitin ligases and of the VHL complex controlling the conjugation of ubiquitin or ubiquitin-like proteins to various sets of target proteins. The hypoxia-inducible transcription factor HIF1 α is a likely target for ubiquitination by the VHL complex (Maxwell *et al* "The tumor suppressor protein VHL targets hypoxi-inducible factors for oxygen-dependent proteolysis" *Nature* 399:271, 1999). Hypoxia-inducible transcription factors regulate the expression of hypoxia inducible genes including vascular endothelial growth factor, which controls normal vascularization as well as vascularization of tumors. Hence, compounds that disrupt or interfere with the function of the VHL complex are likely candidates for anti-cancer drugs or for drugs that promote vascularization.

In another aspect, the present invention provides methods for diagnosing the predisposition of a patient to certain carcinomas. The invention is based upon the discovery that the absence of VHL-associated proteins, i.e., Ring box proteins such as Rbx1, from certain patient tissues or body fluids indicates that the patient is predisposed to certain carcinomas. The method comprises collecting a tissue or body fluid sample from a patient, analyzing the tissue or body fluid for the quantity of Ring box protein in the tissue, and predicting the predisposition of the patient to certain carcinomas based upon the amount of Ring box protein in the tissue or body fluid. Specifically, determination of Rbx1 protein levels in certain tissues permits specific and early, preferably before metastases occur, detection of carcinomas in the patient. Carcinomas that can be diagnosed using the present method include, but are not limited to, clear cell renal carcinoma.

In another aspect, the present invention provides methods for treating any of several enumerated Ring box protein associated carcinomas or augmenting metabolically deficient systems (e.g., fibronectin deposition) in humans and other animals. One method comprises administering a therapeutically effective amount of a compound that enhances or augments *in vivo* the expression of the target Rbx1 gene and enhances the *in vivo* the expression of the Ring box protein to a patient diagnosed as having a Ring box protein associated carcinoma or cellular deficiency and having been diagnosed as deficient in Ring box protein, preferably Rbx1. In a preferred embodiment, the compound is a nucleobase containing a sequence of the nucleic acid sequence encoding the Ring box protein. After administration, the nucleic acid sequence is activated to produce the Ring box protein and increase the amount of the Ring box protein in the cell. Increasing the amount of Ring box protein increases the activity of VHL tumor suppressor activity, ubiquitination, fibronectin deposition, and similar activities. Another method comprises administering a therapeutically effective amount of a Ring box protein to a patient diagnosed as having a Ring box protein associated carcinoma or cellular deficiency and having been diagnosed as deficient in Ring box protein, preferably Rbx1. Carcinomas that can be treated using the present method include, but are not limited to, clear cell renal carcinoma.

In another aspect, the present invention provides methods for evaluating the effectiveness of a therapeutic treatment for Ring box associated carcinomas. The method comprises collecting a tissue or body fluid sample from a patient suffering from a Ring box associated carcinoma and having been subjected to a therapeutic treatment for such carcinoma, determining the amount of Ring box protein in the tissue or body fluid sample, and comparing the determined amount of Ring box protein to a standard indicative of normal Ring box protein levels. The standard can be averages of Ring box protein levels for normal patients but preferably is the level of Ring box protein for the patient being treated before the treatment began. Elevated levels of Ring box protein compared to the standard indicates that the treatment is effective.

Example 1

Use of Rbx1 with SCF^{Cdc4} in *In Vitro* Sic1 Ubiquitination Assay

Rbx1 can be used as a research tool to better understand various complex mechanisms of cell ubiquitination. To test directly the role of Rbx1 in SCF^{Cdc4} function, a standard *in vitro* Sic1 ubiquitination assay that is dependent upon Sic1 phosphorylation and the E2 Cdc4 was employed. (Skowyra, *et al.* 1997. *Cell* 91:209). SCF^{Cdc4} components were co-expressed in insect cells in the presence or absence of mammalian or yeast Rbx1, and complexes were immunopurified through either MYC-tagged Cdc53(MYC-Cdc53) or FLAG-tagged Skp1 (FLAG-Skp1) subunits. Immunopurified SCF^{Cdc4} complexes were supplemented with

phosphorylated Sic1, Cdc4, E1 ubiquitin activating enzyme, ATP, and GST-Ub^{RA} prior to analysis of Sic1 conjugates by immunoblotting. GST-Ub^{RA} forms polyubiquitinated products only poorly, so Sic1 conjugates are integrated into a ladder of bands differing by ~35 kd, the size of GST-Ub^{RA}. Under the reaction conditions used, low but detectable amounts of Sic1-GST-Ub^{RA} conjugates were produced by the SCF^{Cdc4} complex after a 60 minute reaction. In the presence of either yeast or mammalian Rbx1, the accumulation of Sic1-GST-Ub^{RA} conjugates was dramatically increased after 20 minutes, and substantial amounts of higher molecular mass conjugates accumulated after 60 minutes. A large fraction of phosphorylated Sic1 was converted to Sic1-GST-Ub^{RA} conjugates when reactions included either the anti-Cdc53 or anti-FLAG-Skp1 immune complexes; e.g., >95% or Sic1 was converted to conjugates in reactions containing the anti-Skp1 immune complex. In contrast, less than 5% of the substrate was conjugated in the absence of Rbx1 by either Cdc53 or Skp1 immune complexes, despite the presence of larger amounts of Cdc4 or Cdc53. To examine the extent of activation and the concentration dependence of Rbx1 activation, SCF^{Cdc4} complexes were purified from insect cells co-expressing varying levels of MYC-Rbx1 and then assayed for Sic1 ubiquitin conjugating activity. In the absence of Rbx1, low levels of conjugates were observed. Increasing quantities of Rbx1 led to increasing levels of ubiquitination, with the maximal extent of activation approaching 20-fold. This estimate represents a lower limit of the extent to which Rbx1 can increase the rate of accumulation of Sic1-GST-Ub^{RA} conjugates, because a large fraction of the phosphorylated Sic1 substrate was depleted at the end of the reactions. Immunoblot analysis of these complexes revealed that the levels of Cdc53, Cdc4, and Skp1 were constant throughout the Rbx1 titration.

Example 2

Use of Rbx1 with SCF^{β-TRCP} to Stimulate IκBα Ubiquitination

As another example of utilizing Rbx1 as a research tool, Rbx1 can be used to stimulate ubiquitination of phosphorylated IκB in the presence of E1 ubiquitin-activating enzyme, the E2 ubiquitin conjugation enzyme UBC5C, ATP, GST-Ub^{RA}, and SCF^{β-TRCP} (which contains the F-box protein β-TRCP). (Yaron, *et al.* 1998. "Identification of the receptor component of the IκBα-ubiquitin ligase," *Nature* 396:590-594; Winston, *et al.* 1999. "The SCF^{β-TRCP}-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBα and β-catenin and stimulates IκBα ubiquitination in vitro," *Genes Dev* 13:270-283). In a preferred embodiment, complex of Rbx1 and SCF^{β-TRCP} can be purified from lysates of Hi5 or Sf21 insect cells infected with baculoviruses encoding SCF subunits and Rbx1. Complexes can be supplemented with phosphorylated IκB prepared as described by Winston (Winston, *et al.* 1999.

Genes Dev 13:270-283), UBC5C, E1 ubiquitin-activating enzyme, ATP, and GST-Ub^{RA}. After a period of incubation, reaction products can be subject to SDS-PAGE and analyzed by Western blotting using antibodies against IkB α . Conjugation of GST-Ub^{RA} to IkB α can result in the formation of a ladder of IkB α conjugates differing in size by ~35 kD, the size of GST-Ub^{RA}.

- 5 Confirmation that Rbx1 can be used as a research tool has been published, e.g. Tan *et al*, *Mol Cell* 3:527 (1999).

Example 3

Use of Rbx1 with the VHL Complex to Reconstitute HIF1 α Ubiquitination

- As another example of utilizing Rbx1 as a research tool, Rbx1 was used in assays for
10 ubiquitination of HIF1 α (Maxwell *et al* "The tumor suppressor protein VHL targets hypoxi-inducible factors for oxygen-dependent proteolysis" *Nature* 399:271, 1999) in the presence of E1 ubiquitin-activating enzyme, the E2 ubiquitin conjugation enzyme UBC5a, ATP, GST-Ub^{RA}, and a complex consisting of VHL protein, Elongin B, Elongin C, CUL2, and Rbx1. In a preferred embodiment, the VHL-containing complex is purified from Sf21 insect cells infected
15 with baculoviruses encoding subunits of the VHL complex. VHL complexes are supplemented with lysates from insect cells infected with baculoviruses encoding HIF1 or HIF2 and with UBC5a, E1 ubiquitin activating enzyme, ATP, and GST-Ub^{RA}. After a period of incubation, reaction products are subjected to SDS-PAGE and analyzed by Western blotting using antibodies against HIF1 α . Conjugation of GST-Ub^{RA} to HIF1 α results in the formation of a
20 ladder of HIF1 α conjugates differing in size by ~35 kD, the size of GST-Ub^{RA}.

Example 4

Use of Rbx1 to stimulate Rub1 (NEDD8) modification of Cullin proteins

- Rbx1 was used to stimulate modification of the cullin proteins Cdc53 and CUL2 with the ubiquitin-like protein Rub1 (also known as NEDD8) in the presence of Rub1-activating enzyme
25 Uba3/Ul1, Rub1-conjugating enzyme Ubc12, ATP, GST-Rub1, and a complex consisting of Rbx1 and either Cdc53 or Cul2 (Kamura *et al* "The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2" *Genes Dev.* 13:2928, 1999). In one embodiment, the Cdc53-Rbx1 complex is purified from Sf21 cells infected with baculoviruses encoding Cdc53 and Rbx1. Complexes are supplemented with Uba3/Ul1,
30 Ubc12, ATP, and GST-Rub1. After a period of incubation, reaction products are subjected to SDS-PAGE and analyzed by Western blotting using antibodies that recognize Cdc53. Conjugation of GST-Rub1 to Cdc53 results in disappearance of unmodified Cdc53 and appearance of a new, more slowly migrating band corresponding to the Cdc53-GST-Rub1 conjugate. In another embodiment, the CUL2-Rbx1 complex is purified from Sf21 cells
35 infected with baculoviruses encoding CUL2 and Rbx1. Complexes are supplemented with

Uba3/Ul1, Ubc12, ATP, and GST-Rub1. After a period of incubation, reaction products are subjected to SDS-PAGE and analyzed by Western blotting using antibodies that recognize CUL2. Conjugation of GST-Rub1 to CUL2 results in disappearance of unmodified CUL2 and appearance of a new, more slowly migrating band corresponding to the Cdc53-GST-Rub1 conjugate.

Example 5

Reconstitution of the VHL Complex

Reconstitution of the VHL complex and its subassemblies in insect cells and with bacterially expressed proteins *in vitro* shows that Rbx1 can interact with VHL, CUL2, and the Elongin BC complex.

Rbx1 and VHL were subcloned into pBacPAK8 with N-terminal His tags and N-terminal myc and C-terminal FLAG tags, respectively. CUL1 was introduced in to the same vector with a C-terminal HA tag, and CUL2 was introduced into pBacPAK-His1 with N-terminal His and HA tags, and recombinant baculoviruses were generated using the BacPAK baculovirus expression system (Clontech). The baculovirus vectors encoding *S. cerevisiae* CDC53 (Willems, *et al.* 1996. "Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway," *Cell* 86:453-463) and Elongins B and C have been described (Kamura, *et al.* 1998. "The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat FAMILIES," *Genes Dev* 12:3872-3881). Sf21 cells were cultured in Sf-900 II SFM with 5% fetal calf serum at 27°C and coinfectd with various combinations of baculoviruses encoding myc-Rbx1, FLAG-VHL, HA-CUL2, HPC4-Elongin B, and HSV-Elongin C. At 60 hours after infection, Sf21 cells were collected and lysed by gentle vortexing in ice-cold buffer containing 40 mM Hepes-NaOH, pH 7.9, 150 mM NaCl, 1 mM DTT, 0.5% (v/v) glycerol, 5 µg/ml leupeptin, 5 µg/ml antipain, 5 µg/ml pepstatin A, and 5 µg/ml aprotinin. Lysates were centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatants were used for immunoprecipitations.

For the expression and purification of recombinant proteins in *E. coli*, full-length mouse Rbx1 was expressed in pRSET B (Invitrogen) with N-terminal 6-histidine and myc epitope tags. Human VHL was expressed in pRSET B with N-terminal 6-histidine and C-terminal FLAG epitope tags. Purification of recombinant proteins from inclusion bodies and expression constructs for Elongins B and C have been described previously. (Kamura, *et al.* 1998. *Genes Dev* 12:3872-3881).

The procedures for immunoprecipitations and Western Blotting are as follows. Anti-T7 and anti-HSV antibodies were from Novagen. Anti-HA (12CA5) and anti-c-myc (9E10) antibodies were from Boehringer-Mannheim. Anti-FLAG (M2) was from Eastman Kodak.

Anti-Elongin C monoclonal antibody was from Transduction Laboratories. Anti-VHL monoclonal antibody (Ig32) was from Pharmingen. Anti-Sic1 (yN-19 and yC-19) and anti-Cdc53 (yC-17) antibodies were from Santa Cruz Biotechnology, Inc. Anti-Elongin B rabbit polyclonal antibodies were described previously. (Garrett, *et al.* 1995. "Positive regulation of
5 general transcription SIII by a tailed ubiquitin homolog," *Proc Natl Acad Sci USA* 92:7172-7176). Anti-HPC4 monoclonal antibody (Stearns, *et al.* 1988. "The interaction of a Ca^{2+} -dependent monoclonal antibody with the protein C activation peptide region. Evidence for obligatory Ca^{2+} binding to both antigen and antibody," *J Biol Chem* 263:826-832) was obtained from a colleague. Western blots and immunoprecipitations of insect cell lysate and refolded
10 bacterially expressed proteins were performed as described. (Kamura, *et al.* 1998. *Genes Dev* 12:3872-3881).

As illustrated in Fig. 3A, all five components of the VHL complex could be coimmunoprecipitated together from Sf21 cell lysates with either anti-FLAG (Lane 5) or anti-myc (Lane 9) antibodies. In addition, Rbx1, VHL, and the Elongin CB complex could be
15 coimmunoprecipitated from Sf21 cell lysates with either anti-FLAG (Lane 6) or anti-myc (Lane 10) antibodies in the absence of CUL2; and Rbx1, CUL2, and Elongin BC complex could be coimmunoprecipitated from Sf21 cell lysates with anti-myc antibody in the absence of VHL (Lane 11). As shown in Fig. 3B and 3D, Rbx1 could also be coimmunoprecipitated with anti-myc antibody with either VHL, CUL2, or the Elongin BC complex from lysates of Sf21 cells
20 coinfecting with either myc-Rbx1 and FLAG-VHL, myc-Rbx1 and HA-CUL2, or myc-Rbx1, HPC4-Elongin B, and HSV-Elongin C. Consistent with these results, as shown in Fig. 3C, Rbx1 could be coimmunoprecipitated with VHL-Elongin BC and Elongin BC subassemblies reconstituted *in vitro* with bacterially expressed FLAG-VHL, HPC4-elongin B, and HSV-Elongin C.

25 CUL2 is a member of the multiprotein Cullin family (Kipreos, *et al.* 1996. "Cul-1 is required for cell cycle exit in *C. elegans* and identifies a novel gene family," *Cell* 85:829-839), which also includes the SCF component CUL1 and its *S. cerevisiae* homolog Cdc53. Because Rbx1 interacted with CUL2 as shown in Fig. 3A, the possibility that it might also interact with CUL1 and Cdc53 was examined. As shown in Fig. 3D, Rbx1 binds both human CUL1 and *S.*
30 *cerevisiae* Cdc53. Myc-Rbx1 and human HA-CUL1 could be coimmunoprecipitated with anti-myc antibodies from lysates of Sf21 cells coinfecting with baculoviruses encoding myc-Rbx1 and HA-CUL1. In addition, myc-Rbx1 and Cdc53 could be coimmunoprecipitated with anti-myc antibodies from lysates of Sf21 cells coinfecting with baculoviruses encoding myc-Rbx1 and Cdc53.

Example 6

Effect of Rbx1 on Function of SCF^{Cdc4} Ubiquitin Ligase

Cdc53/CUL1 is a component of the recently described SCF ubiquitin ligase complex, which catalyzes ubiquitination of a diverse collection of proteins with critical roles in cell cycle, transcription, and development. (Patton, *et al.* 1998. "Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complex that regulate cell division and methionine biosynthesis in yeast," *Genes Dev* 12:692-705; Bai, *et al.* 1996. "SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box," *Cell* 86:263-274; Patton, *et al.* 1998. "Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis," *Trends Biochem Sci* 14:236-243; Skowyra, *et al.* 1997. "F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex," *Cell* 91:209-219; and Feldman, *et al.* 1997. "A complex of Cdc4p, Sklp, and Cdc53p/Cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p," *Cell* 91:221-230). SCF complexes include Cdc53/CUL1, Skp1, and one of the variety of F-box proteins, which recruit substrates to the SCF for ubiquitination. (Patton, *et al.* 1998. *Genes Dev* 12:692-705; and Bai, *et al.* 1996. *Cell* 86:263-274). The results demonstrate that Rbx1 can assemble into complexes containing CUL1 and the additional SCF component Skp1, since Myc-Rbx1 could be co-immunoprecipitated with anti-T7 antibodies from lysates of Sf21 cells coinfecting with viruses encoding myc-Rbx1, T7-tagged Skp1 (T7-Skp1), and HA-CUL1.

The WD40 repeat protein Cdc4, which recruits the cdk inhibitor Sic1 for ubiquitination by the SCF^{Cdc4} complex, and the leucine-rich repeat protein Grr1, which recruits G1 cyclin Cln2 for ubiquitination by the SCF^{Grr1} complex, are among several F-box proteins found in yeast. Having shown that Rbx1 interacts in cells not only with CUL2 and the VHL complex but also with the SCF components Cdc53/CUL1 (Fig. 3A) and Skp1, mutant yeast strains lacking the chromosomal *RBX1* gene were generated in order to test the possibility that Rbx1 might affect functions of SCF^{Cdc4} and SCF^{Grr1}.

A *S. cerevisiae* strain lacking the Rbx1 gene was constructed as follows. The Rbx1 gene was disrupted in MCY453 (Mata/MAThis3)-200/his3)-200 can1R/can1R cyh2Rcyh2R ura3/ura3 leu2/leu2 trp1/trp1 lys2/lys2) by replacing the complete Rbx1 ORF (YOL133w) with the HIS3 gene (Baudin, *et al.* 1993. "A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*," *Nucleic Acids Res* 21:3329-3330). For rescue of the rbx1 deletion strain (MCY557) with mammalian RBX1, wild type and mutant mammalian RBX1 genes were fused to the GAL1,10 promoter in the plasmid Yep352-GAL. These plasmids were transformed into MCY557 and URA3+ transformants were selected. Random spores were germinated on galactose medium minus histidine and uracil and allowed to germinate for 4 days at 30°C. The

resulting colonies were tested for mating. To confirm that rescue was due to the presence of the SCF4 expression plasmid, cells were tested for the ability to grow in FOA after prolonged growth in medium containing uracil. Sporulation and tetrad dissection showed 2:0 segregation for viability, and all viable spores were HIS⁺, indicating the *RBX1* is an essential gene. In viable spores produced microcolonies of 10-20 cells, many of which were abnormally elongated or contained multiple, abnormally shaped buds. *S. cerevisiae* strains containing mutations in genes encoding the SCF components Cdc53, Skp1, Cdc4, and Cdc34 exhibit a similar morphology. This phenotype was due to the RBX1 deletion, because the *rbx1* deletion strain could be rescued by expression of *yRbx1*.

The *Rbx1* deletion strain was also rescued by expression of either myc-tagged mammalian *Rbx1* (mRbx1) or a mutant mRbx1 (M4), in which putative ring finger cysteine 53 and cysteine 56 were replaced with serines. The *rbx1* deletion strain was not rescued, however, by expression of a mutant mRbx1 (M3), in which putative ring finger cysteine 42 and cysteine 45 were replaced by serines. When expressed in either the *rbx1* deletion strain or in a wild type background, myc-tagged mRbx1 coimmunoprecipitated with the endogenous yeast Cdc53 protein, suggesting that it associates with Cdc53 in cells (Fig. 4A). Furthermore, coimmunoprecipitation of wild type and mutant mRbx1 proteins with Cdc53 correlated with their abilities to rescue the deletion phenotype, since significantly more Cdc53 was coimmunoprecipitated with the complementing mRbx1 M4 mutant than with the noncomplementing M3 mutant (Fig. 4A).

These results show that yeast *Rbx1* (*yRbx1*) is a subunit and activator of the SCF^{Cdc4} ubiquitin ligase and that mammalian *Rbx1* (mRbx1) can substitute for its yeast counterpart in reconstitution of the active SCF^{Cdc4} complex.

The SCF^{Cdc4} complex is required for ubiquitination and targeted degradation of a variety of cell cycle regulatory proteins including Sic1, whose degradation is essential for the G1/S transition. The SCF^{Gm1} complex is required for ubiquitination and targeted degradation of the G1 cyclin Cln2. As one means of addressing the role of *Rbx1* in these processes, myc-tagged wild type or M4 mutant mRbx1 were expressed in the *rbx1* deletion strain on a high copy number plasmid under control of the GAL1,10 promoter. When cells carrying the plasmid were shifted from galactose medium to glucose medium, *Rbx1* protein was depleted, and the fraction of cells exhibiting the elongated bud morphology increased dramatically (Fig. 4C). Cells expressing the M4 mutant arrested growth within a few hours of the glucose shift, whereas cells expressing wild type *Rbx1* continued to grow slowly, presumably due to the presence of a small amount of residual *Rbx1*. As expected if M4 cells arrest at least in part due to their inability to

ubiquitinate the cell cycle regulators Sic1 and Cln2, M4 cells accumulated Sic1 and Cln2 proteins when shifted into glucose (Fig. 4B).

Showing that Rbx1 is a general subunit of SCF ubiquitin-ligases demonstrates (1) that Rbx1 interacts with subunits of Cdc53/CUL1 containing SCF ubiquitin-ligase complexes, (2) that the abilities of Rbx1 mutants to bind the Cdc53 subunit of SCF ubiquitin ligase correlate with their abilities to prevent cell cycle arrest in a yeast strain lacking chromosomal Rbx1, and (3) that depletion of Rbx1 from yeast interferes with the function of SCF^{Cdc4} and SCF^{Grr1}. Having shown that Rbx1 is also a component of the CUL2-containing VHL complex, Rbx1 can now be applied in delineating the function of the VHL complex and perhaps other Cullin complexes as possible ubiquitin ligases and ubiquitin-like ligases for as yet unidentified sets of target proteins. Since the multiprotein VHL complex has been shown to have roles in cell cycle regulation through its control of the levels of the cdk inhibitor p27, in repression of hypoxia-inducible genes, and in assembly of the extracellular fibronectin matrix, Rbx1 can be applied in the reconstitution of VHL complexes to determine therapeutics that cause the dissociation of the VHL complex. Further, Rbx1 is a component of the SCF^{Cdc4} complex, functioning as a common SCF subunit and participating in regulation of ubiquitination by SCF complexes containing additional F-box proteins, including MET30 and β -TRCP, which direct ubiquitination of such target proteins as Swi6 and the transcriptional regulators I κ B and β -catenin. Thus, Rbx1 can be applied in determining therapeutics capable of regulating ubiquitination via other SCF complexes.

The present invention relates to a component in VHL tumor suppressor activity found in the majority of sporadic clear cell renal carcinomas as well as autosomal dominant familial cancer syndrome that predisposes affected individuals to a variety of tumors including clear cell renal carcinomas, cerebellar hemangioblastomas and hemangiomas, retinal angiomas, and pheochromocytomas; general repression of hypoxia-inducible genes; and regulation of p27 protein stability and fibronectin matrix assembly. The Ring box proteins described act as a cellular marker useful (1) in detecting a possible predisposition to certain carcinomas, (2) as molecular targets for treating those carcinomas therapeutically, (3) as a target for inhibition by drugs that manipulate the growth of cells, and (4) as a research tool to better understand the various complex mechanisms of cell ubiquitination, binding of certain activator proteins, fibronectin deposition and other aspects of the cellular division process.

While the preferred embodiments are shown to illustrate the invention, numerous changes to the materials and methods can be made by those skilled in the art. All such changes are encompassed within the spirit of the invention as defined by the appended claims.

What is claimed is:

1. An isolated and purified biologically active Ring box protein comprising a polypeptide having an amino acid sequence corresponding to SEQ ID NO:1.
2. An isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding a biologically active Ring box protein having an amino acid sequence corresponding to SEQ ID NO:1.
3. The nucleic acid molecule of Claim 2 comprising SEQ ID NO:3.
4. An expression vector containing the nucleic acid molecule of any one of Claims 2 or 3.
5. A host cell containing the expression vector of Claim 4.
6. A method for producing recombinant biologically active Ring box proteins comprising the steps of:
 - culturing a host cell of Claim 5 under conditions suitable for expression of the nucleic acid molecule; and
 - recovering the recombinant biologically active Ring box protein from the host cell culture.
7. A recombinant Ring box protein prepared according to the method of Claim 6.
8. A protein complex useful for screening for potential therapeutic agents that would interfere with or augment Rbx1-dependent stimulation of addition of ubiquitin or a ubiquitin-like protein to any substrate targeted for modification by SCF complexes comprising a cofactor protein and one or more proteins selected from the group consisting of a cullin protein, a substrate recognition protein, and a linker protein.
9. The protein complex of Claim 8 wherein the complex is a ubiquitin ligase protein complex.
10. The protein complex of Claim 9 wherein the ubiquitin ligase protein complex is selected from the group consisting of SCF and VHL.
11. The protein complex of Claim 8 wherein the cofactor protein is Rbx1.
12. An isolated and purified ubiquitin ligase protein complex, comprising:
 - a cullin protein;
 - a substrate recognition protein;
 - a linker protein; and
 - a cofactor protein.
13. The ubiquitin ligase protein complex of Claim 12 wherein the cullin protein is selected from the group consisting of Cdc53, Cullin 1, Cullin 2, Cullin 3, Cullin 4A, Cullin 4B, and Cullin 5.

14. The ubiquitin ligase protein complex of Claim 12 wherein the substrate recognition protein is selected from the group consisting of β -TRCP, Cdc4, Grr1, VHL and Elongin C binding proteins.
15. The ubiquitin ligase protein complex of Claim 12 wherein the linker protein is selected from the group consisting of Skp1 and Elongin C.
16. The ubiquitin ligase protein complex of Claim 12 wherein the cofactor protein is Rbx1.
17. A method for screening for potential therapeutic agents that would interfere with or augment Rbx1-dependent stimulation of addition of ubiquitin or a ubiquitin-like protein to any substrate, comprising:
 - 10 forming a complex *in vitro* that contains a cofactor protein and one or more proteins selected from the group consisting of a cullin protein, a substrate recognition protein, and a linker protein;
 - adding a test compound to interact with the complex; and
 - determining if the complex remains intact or is disrupted by the compound.
- 15 18. The method of Claim 17 wherein the cofactor protein is Rbx1.
19. A method for diagnosing a predisposition of a patient to certain carcinomas, comprising collecting a tissue or body fluid sample from a patient; analyzing the tissue or body fluid for the quantity of Ring box protein in the tissue; and predicting the predisposition of the patient to certain carcinomas based upon the amount of Ring box protein in the tissue or body fluid.
- 20 20. A method for treating Ring box protein associated carcinomas or augmenting metabolically deficient systems in animals comprising administering to a patient diagnosed as having a Ring box protein associated carcinoma or cellular deficiency and having been diagnosed as deficient in Ring box protein a therapeutically effective amount of a compound that enhances or augments *in vivo* the expression of the Rbx1 gene and enhances the *in vivo* the expression of the Ring box protein.
- 25 21. A method for treating Ring box protein associated carcinomas or augmenting metabolically deficient systems in animals comprising administering a therapeutically effective amount of a Ring box protein to a patient diagnosed as having a Ring box protein associated carcinoma or cellular deficiency and having been diagnosed as deficient in Ring box protein.
- 30 22. A method for evaluating the effectiveness of a therapeutic treatment for Ring box associated carcinomas, comprising

- collecting a tissue or body fluid sample from a patient suffering from a Ring box associated carcinoma and having been subjected to a therapeutic treatment for such carcinoma;
- determining the amount of Ring box protein in the tissue or body fluid sample; and
- 5 comparing the determined amount of Ring box protein to a standard indicative of normal Ring box protein levels.
23. The method of Claims 19, 20, 21, and 22 wherein the Ring box protein is Rbx1.

Fig. 1

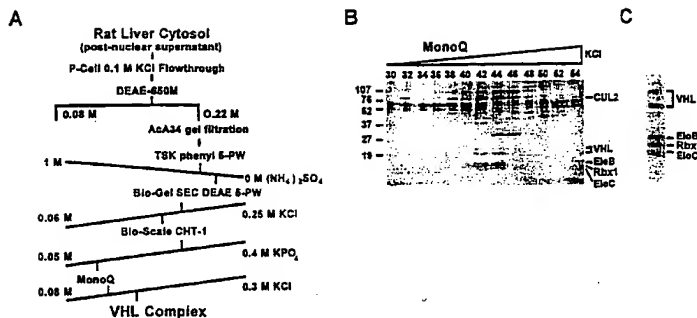


Fig. 2

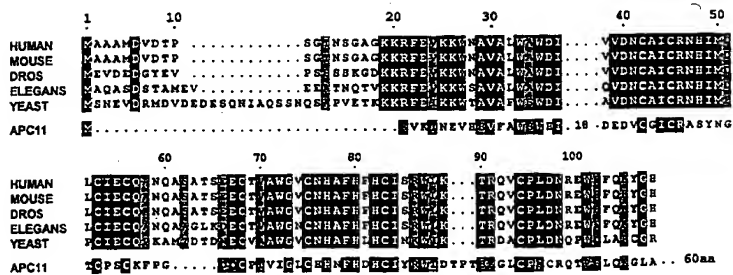


Fig. 3

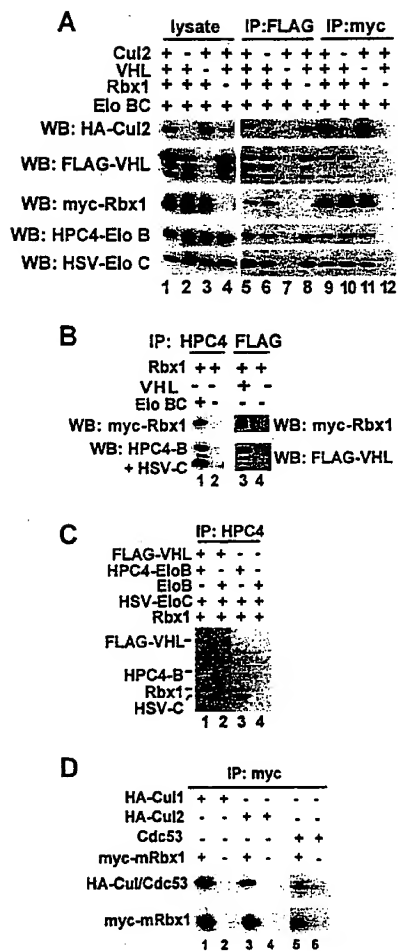
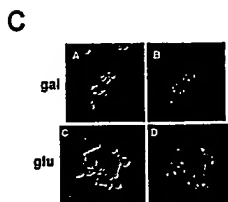
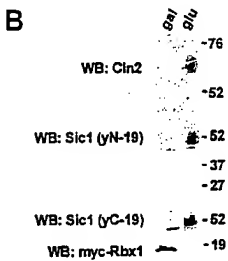
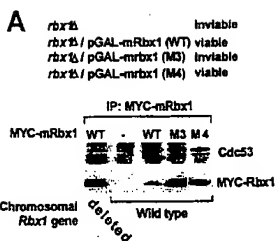


Fig. 4



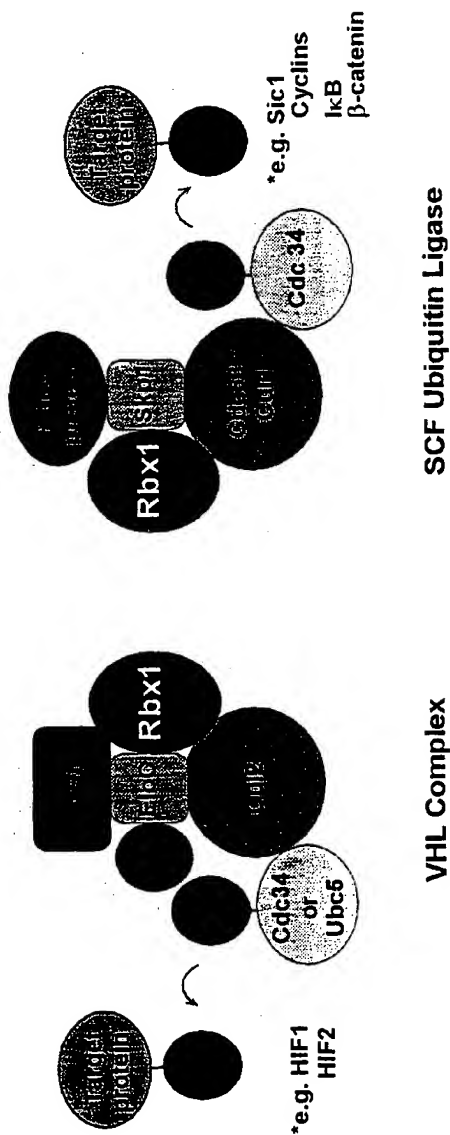


FIG. 5

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KAMURA, Takumi
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04838

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : 530/350; 435/320.1; 435/325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 435/320.1; 435/325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN. EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALLIEL, P.M., et. al. Testican, a multidomain testicular proteoglycan resembling modulators of cell social behavior. <i>European Journal of Biochemistry</i> . 1993, Vol. 241, No. 1, pages 347-350, especially page 347.	1
A	OHTA, T., et. al. ROC1 a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity, <i>Mol. Cell</i> . April 1999, Vol. 3, No. 4, pages 535-541, especially page 535.	1-23
A	KAMURA, T. et. al. Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. <i>Science</i> . Vol. 284, No. 5414, 23 April 1999, pages 657-661, especially page 657.	1-23

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z

document member of the same patent family

Date of the actual completion of the international search

22 MAY 2000

Date of mailing of the international search report

04 AUG 2000

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Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04838

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C07K 1/00, 14/00, 17/00; A21C 3/06, 3/08; B28B 11/08, 21/48, 21/98; B29C 53/00, 53/14; C12N 5/00, 5/02